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The RNA-binding protein *XSeb4R* regulates maternal *Sox3* at the posttranscriptional level during maternal-zygotic transition in *Xenopus*

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ABSTRACT

The maternal-zygotic transition (MZT) is an embryonic event that overlaps with and plays key roles in primary germ layer specification in vertebrates. During MZT, maternally supplied mRNAs are degraded while zygotic transcripts are synthesized to either reinforce the already specified cell fate or to trigger new cell identity. Here, we show that forced expression of the RNA-binding protein, *XSeb4R*, in animal pole blastomeres of *Xenopus* embryos, inappropriately stabilizes transcripts there, including maternal *Sox3*. This leads to the impaired ability of the ectodermal progenitors to respond to factors regulating brain patterning and their eventual loss by apoptosis. XSeb4R protein binds specifically to the 3′UTR of *Sox3* mRNA. *XSeb4R* gain-of-function in ectodermal explants reveals increased stability of the maternal *Sox3* transcripts, associated with a robust Sox3 protein production. Conversely, whereas *XSeb4R* depletion abolishes *VegT* expression, the amount of the maternal *Sox3* mRNA is rather increased but without augmentation in the amount of Sox3 protein. Moreover, XSeb4R protein knockdown leads to the modification of the ectoderm–mesoderm boundary, marked by expanded/shifted expression of the mesodermal marker genes such as *Xbra* and *Apod*, followed by an expression inhibition of *Epi. K.*, an ectodermal marker. Overall, our data suggest *XSeb4R* as a novel player in gene expression regulation, acting at the posttranscriptional level during ectoderm specification in *Xenopus*.

Introduction

During vertebrate embryonic development, gastrulation gives rise to three germ layers: the ectoderm, mesoderm and endoderm. In the *Xenopus* embryo, some maternally supplied determinants are asymmetrically distributed along the animal–vegetal axis and promote germ layer specification (Heasman, 2006). For example, the vegetally localized T-box transcription factor *VegT* is a master regulator of endoderm specification and mesoderm induction in *Xenopus* (Stennard et al., 1996; Zhang and King, 1996; Zhang et al., 1998). It initiates cascades of gene transcription and signaling pathways to drive vegetal blastomeres into endodermal fate (Clements et al., 1999; Sinner et al., 2006; Xanthos et al., 2001). *VegT* downstream transcriptional targets, of the *TGF-β/Nodal* superfamily, in turn induce the overlying cells of the embryonic equator to undergo mesodermal fate differentiation (Kofron et al., 1999; Piccolo, 2008).

The early animal pole cells of *Xenopus* embryos are pluripotent and permissive to instructive factors, such as the mesodermalizing signals, Nodal/Xnrs (Schier, 2003). Based on these characteristics the animal pole derived explants of amphibian embryos gave rise to the so-called animal cap system, which has been intensively used to investigate various signal transduction and gene function (Green, 1999; Sasai et al., 2008b). The molecular mechanisms underlying the pluripotency of these progenitors are not well understood. Moreover, it remains unclear how the boundary between the induced mesoderm, enriched in secreted TGFβ/Nodal signals, and the prospective ectoderm is established (De Robertis and Kuroda, 2004; Niehrs, 2004).

It is becoming evident that maternal ectodermal determinants restrict mesoderm differentiation and this pattern is reinforced later by specific zygotic factors (Piccolo, 2008). *Ectodermin*, a RING-type E3 ubiquitin ligase, was the first key ectoderm instructor characterized. It acts by triggering the degradation of Smad4, thereby attenuating animal cap cells responsiveness to TGFβ signals (Dupont et al., 2005). *Coco*, a secreted molecule, was reported to be able to inhibit TGFβ responses in the developing ectoderm (Bell et al., 2003). In addition, another maternally expressed factor, the B1-type HMG Sox protein, *Sox3*, was suggested to negatively modulate TGFβ signals and to activate *Ectodermin*, *Foxl1e/Xema* and *Coco* expression (Zhang and Klymkowsky, 2007).

Besides these maternally provided determinants, several zygotic factors play crucial roles in ectoderm formation. The Zn-Finger

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protein, *XFDL156*, interacts with the C-terminal regulatory region of p53, another regulator of TGFβ responses, thereby inhibiting its transcriptional activities and preventing mesodermal differentiation in the presumptive ectoderm (Sasai et al., 2008a). Finally, the forkhead protein *FoxI1e/Xema*, is exclusively expressed in the animal pole of blastula stage embryos and was shown to be required in ectoderm formation by a mechanism as yet unclear (Mir et al., 2007; Suri et al., 2005).

Overall, primary ectoderm formation requires timely and spatially coordinated actions of both the maternal and zygotic determinants. During embryonic development, maternally provided mRNAs must be systematically degraded while zygotic players take over the cell specification and differentiation activities. This process, known as the maternal-zygotic transition (MZT), is regulated by microRNAs and RNA-binding proteins (Schier, 2007). We recently showed that *VegT* mRNA translation and stability were positively regulated by the RNA-binding protein *XSeb4R* (Souopgui et al., 2008). In this study, we unveil maternal *Sox3* regulation at the posttranscriptional level by *XSeb4R* and highlight a crucial role of such regulation in primary ectoderm specification in *Xenopus*.

Results

The timing of maternal XSeb4R transcripts degradation precedes that of maternal Sox3 mRNA in Xenopus embryos

During MZT, the kinetics of maternal mRNAs degradation varies from one transcript to another (Schier, 2007). Accordingly, the expression characteristics of *XSeb4R* and *Sox3* were compared by wholemount *in situ* hybridization (WMISH). Results obtained confirmed that these genes are expressed maternally (Penzel et al., 1997; Souopgui et al., 2008) and are co-expressed in the animal hemisphere of blastula embryos (Figs. 1A and E). As development proceeds, signals corresponding to these inherited transcripts decline sequentially. At the early gastrula stage, the maternal *XSeb4R* signal fades out while that of *Sox3* remains unchanged. In the mean time, zygotic *XSeb4R* expression arises in the developing mesoderm around the blastopore (Figs. 1B and F). By mid-gastrula stage, maternal *Sox3* signal, in its turn, fades out and is replaced by zygotic expression at the forming neural plate on the dorsal side of the embryo while *XSeb4R* zygotic expression expands in the mesoderm (Figs. 1C and G). These zygotic spatial expression patterns further diverge at the open neural plate stage (Figs. 1D and H). To gain more insight into this differential timing as well as the kinetics of maternal RNA degradation, a time-course analysis was performed using ectodermal explants coupled to real-time RT-PCR (Suppl. Figs. S1A–D). The treatment of cap explants for five hours with α-amanitin (Lee et al., 2001; Skirkanich et al., 2011), an inhibitor of RNA polymerase II, to inhibit zygotic transcription led to reduced levels of *XSeb4R* and the control zygotic gene, *Epi. K.*, but not *Sox3* transcripts compared to the naive caps (Suppl. Fig. S1E), suggesting zygotic transcriptional activation of *XSeb4R* but not *Sox3* in naive cap explants. These results could not help to highlight more conveniently the difference in *Sox3* and *XSeb4R* mRNA degradation kinetics. Nevertheless, the timing of expression characteristics, interpreted by the diagram in Fig. 1J, matches the MZT process (Schier, 2007) and fulfills the primary requirements for *XSeb4R* as a potential posttranscriptional regulator of *Sox3* expression in *Xenopus* embryos.

XSeb4R-overexpressing cells positively respond to Sox3 expression but are later eventually lost by apoptosis

To investigate whether *Sox3* expression could be regulated by *XSeb4R*, embryos overexpressing *XSeb4R* upon mRNA injection into the animal pole blastomeres were analyzed by WMISH, using a *Sox3* probe. As results, forced expression of *XSeb4R* led to strong ectopic *Sox3* expression in neurula stage embryos (Fig. 1I). This was associated with increased cell proliferation (Fig. 1K) and an expression inhibition of *Epi. K.*, an epidermal marker, as well as *N-tubulin*, a neuronal marker gene (Figs. 1L, M). As zygotic *Sox3* expression marks neurally differentiated cells, we hypothesized that more neural tissues will be formed if these ectopic *Sox3*-positive cells were irreversibly fated. To our surprise, a massive set of pigmented cells (Fig. 2B), negative for *Sox3* after bleaching and WMISH (Fig. 2K), developed at the sites harboring *XSeb4R*-overexpressing cells in tailbuds, marked by X-gal staining (Suppl. Fig. S2A). The pattern of this strong pigmentation (Fig. 2B) correlates with the clustering of the initial *XSeb4R*-overexpressing ectodermal progenetors that might have failed to undergo epiboly, thereby suggesting a *de novo* ectoderm formation which appears unpigmented in a complementary pattern (Fig. 2B compared to Fig. 2A, see posteror-half region of the embryo). At tadpole stage of development, the patches of pigmented cells disappeared and without ectopic melanocyte formation. Instead, embryos revealed important defects in head structure formation (Fig. 2E, see arrow heads).

Such pigmentation patterns were reported in overexpression of the members of ZIC transcription factor family and were shown to be the results of neural crest (NC) cell fate induction followed by ectopic melanocyte differentiation (Nakata et al. 1997, 1998, 2000; Kuo et al., 1998). We have therefore analyzed *XSeb4R*-injecetd embryos with early NC and melanocyte marker genes. As results, we found that *Slug* expression was signficantly repressed in *XSeb4R*-overexpressing cells (84%, n = 45; Figs. 2G and H). Consistently, signals corresponding to *XlTyr* expression, a melanocyte specific marker gene (Kumasaka et al., 2003), was strongly reduced in the site associated with retinal tissue formation (76%, n = 46; Fig. 2I; see black arrow). Conversely, *XSeb4R*-morphant embryos showed no significant changes in the expression extend of these NC and melanocyte markers but rather an important delay of NC cell migration, marked by the clustering of *Slug* expression signals (100%, n = 32; Suppl. Fig. S2B and data not shown).

Moreover, *XSeb4R*-injected embryos showed expanded *Sox3* expression in the brain but revealed no morphological anterior–posterior (A–P) pattern, marked by the absence of a forebrain–midbrain boundary as well as detectable distinct eye fields (Fig. 2, compare J and K, red arrows). Consistently, the expression of *Pitx2*, *Otx2* and *Krox20*, marker genes of the cement gland, forebrain and hindbrain respectively were all strongly suppressed (Figs. 2M, N, O; see red arrows). The RNA recognition motif (RRM) of *XSeb4R* was required in these phenotypes (Fig. 2; compare C, F and L to A, D and J). Finally, TUNEL assays revealed mild increased apoptosis at neurula stage of development (Fig. 2P) but massive ectopic apoptotic patterns at the sites containing *XSeb4R*-overexpressing cells in tailbud stage embryos (Figs. 2R and S), a phenotype which could explain the defects in head structure shown in Fig. 2E.

XSeb4R protein binds specifically to the 3′untranslated region (UTR) of Sox3 mRNA

We tested by UV-crosslinking assays whether XSeb4R protein could bind directly to the *Sox3* transcripts. To this end, we expressed and purified GST-XSeb4R and GST-XSeb4RΔRRM from bacteria (Fig. 3A), as well as Flag-XSeb4R and Flag-XSeb4RΔRRM from HEK293 cells (Suppl. Fig. S3A). As shown in Fig. 2C, XSeb4R protein binds specifically to the 3′UTR of *Sox3* mRNA. Under the same conditions, a *GAPDH* probe showed a very low binding affinity to XSeb4R (Fig. 3C, lane 10). The mutant protein, GST-XSeb4RΔRRM, failed to interact with the *Sox3* 3′UTR probe (Fig. 3D; Suppl. Fig. S3B). With an aim to delineate the minimal sequence to which XSeb4R protein binds, probes were generated as illustrated in Fig. 3B and analyzed. The recombinant XSeb4R protein preferentially binds with the highest

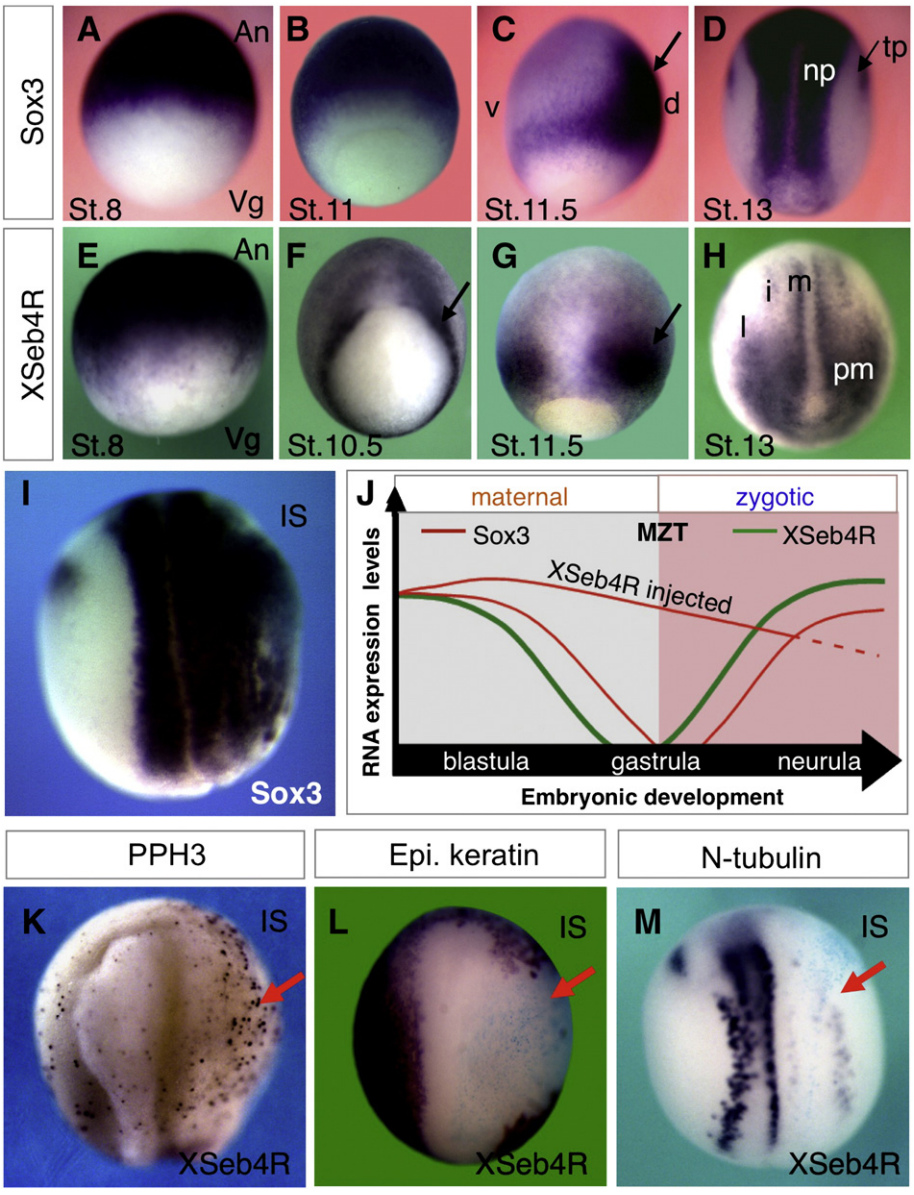

Fig. 1. Perturbed *Sox3* maternal mRNA degradation upon *XSeb4R* overexpression leads to increased cell proliferation and inhibition of cell differentiation. Compared expression patterns of *XSeb4R* and *Sox3* were monitored by wholemount *in situ* hybridization (WMISH), using *Xenopus* albino embryos at the indicated stages (St.) of development. Signals corresponding to these factors (black color) overlap in the animal pole derived cells of blastula embryo, oriented animal pole (An) up and vegetal pole (Vg) down (A, E). These maternal mRNA signals decline sequentially at gastrula stage (B and F, C and G). At these stages, signals of zygotic mRNAs are indicated with arrows; in panel C the embryo is oriented dorsal (d) right and ventral (v) left; in panels F and G the embryos are oriented dorsal side in front and the zygotic *XSeb4R* mRNA signal marks the differentiating mesoderm tissue. Zygotic *Sox3* mRNA signal delineates the neural plate (np) and the trigeminal placodes (tp) at open neural plate stage, oriented dorsal side in front (D). At this stage, *XSeb4R* signals appear, in addition to the presomitic mesoderm (pm), in primary neuron precursors: medial, m (motor neuron); intermediate, i (interneuron) and lateral, l (sensory neuron). The timing of these expression characteristics as well as the phenotype described below are schematically illustrated in panel J. *Capped-XSeb4R* RNA was injected into two micromeres (200 pg each) of 4- or 8-cell stage embryos and analyzed by WMISH at neurula stage using *Sox3* probe. *XSeb4R* overexpression leads to: robust ectopic *Sox3* expression (I, 100%, n = 80); suppression of an epidermal marker (L, 100%, n = 56); suppression of expression (red arrows) of a neuronal marker gene *N-tubulin* (M, 100%, n = 50). Immunostaining of *XSeb4R*-injected embryos using an anti-phosphohistone H3 antibody revealed increased proliferation (red arrow; K, 63%, n = 76). In panels K, L and M embryos are oriented anterior side up with the injected side (IS) on the right. The blue staining (tracer) corresponds to the distribution of *XSeb4R* injected RNA marked by Xgal/LacZ enzymatic reaction.

avidity to UTR-F1b in position 1109–1223 (Figs. 3C and E). The sequence alignment of *Sox3* UTR-F1b and *VegT* UTR-F8 (Souopgui et al., 2008) to which XSeb4R protein binds did not reveal any consensus sequence (data not shown). We further tested if these factors interact *in vivo*. For this, RNA immunoprecipitation (RIP) assays followed by RT-PCR were performed on lysates from cap explants dissected at blastula stage from uninjected embryos as well as those injected with *MT-XSeb4R* or *MT-XSeb4RΔRRM* RNAs. This approach shows that *Sox3* and *VegT* but not *GAPDH* mRNAs are strongly enriched in *XSeb4R*-animal cap explants compared to control samples (Fig. 3F, lane 2), suggesting that XSeb4R also interacts with these target mRNAs *in vivo*.

XSeb4R regulates the stability of maternal Sox3 mRNA in animal cap explants

To address the biochemical relevance of XSeb4R binding to *Sox3* mRNA, we further tested if *XSeb4R* stabilizes maternal *Sox3* mRNA as suggested in Figs. 1I–J. To this end, the animal cap approach, as illustrated in Fig. 4A, was employed. The levels of *Sox3* transcripts

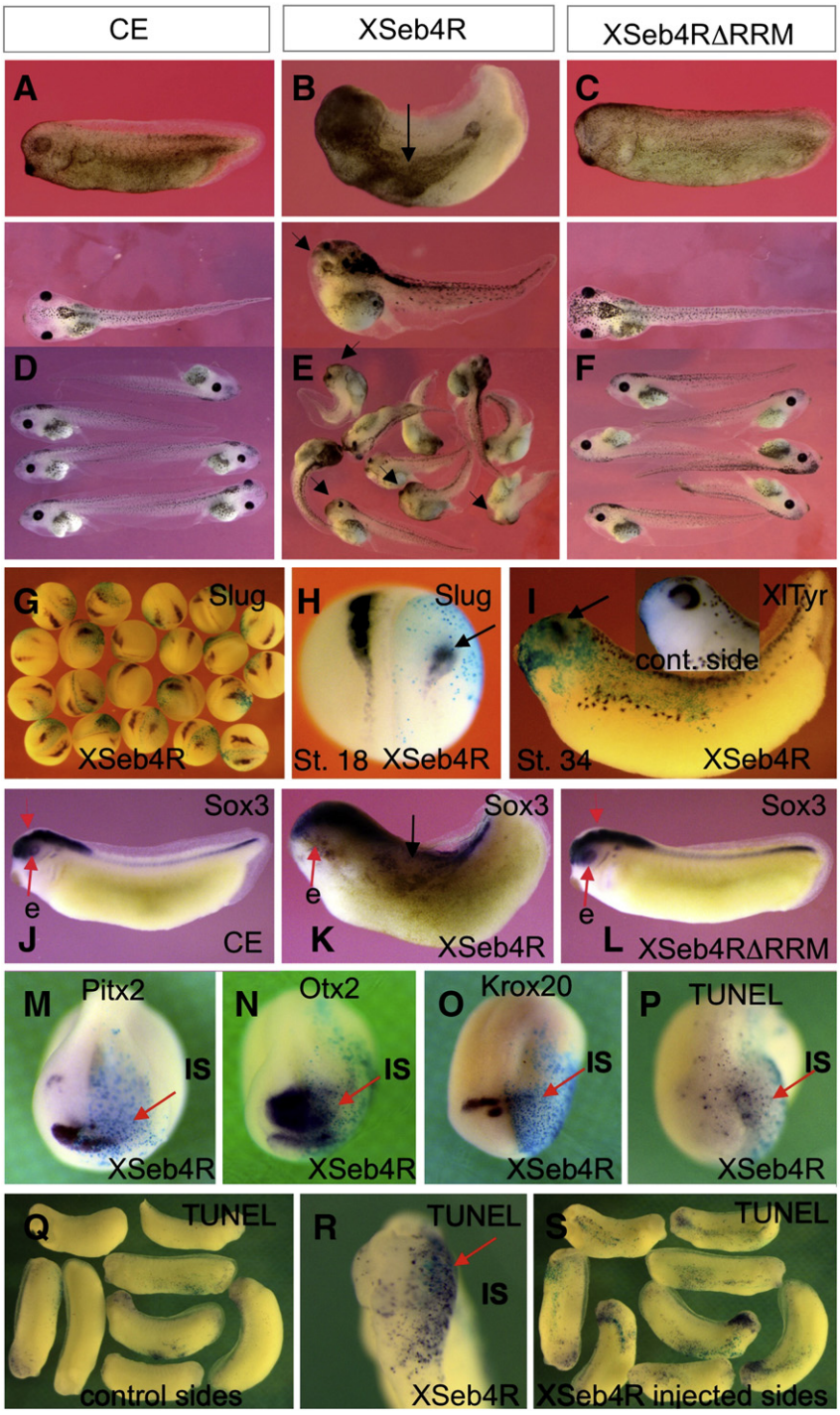

Fig. 2. *XSeb4R* overexpression blocks anterior–posterior (A–P) patterning and activates apoptosis, leading to loss of head structures. Pigmented *Xenopus* embryos were injected with 200 pg of *XSeb4R* or *XSeb4RΔRRM* capped-RNA in each animal blastomere at 4/8 cell stage. Embryos were cultivated to tadpole stage. Compared to control embryos (A) *XSeb4R*-injected one (B) but not *XSeb4RΔRRM*-injected embryo (C) showed ectopic pigmentation pattern (see arrow). This phenotype was associated to a loss of head structures clearly visible (see arrow heads) in later stages of development (compare E to D and F). *XSeb4R*-unilaterally-injected embryos were processed by WMISH using *Slug* (G and H) and *XITyr* (I), marker of neural crest and melanocytes, respectively. Note expression inhibition of these genes in the sites of XSeb4R orverexpression marked by X-gal staining in blue. Embryos shown in A, B and C were bleached and analyzed by WMISH using *Sox3* probe. As shown in K, the pigmented cells were not *Sox3* positive (see black arrow). Brain anterior-posterior patterning, as well as eye structures (red arrow) seen in J and L are not observed in *XSeb4R*-injected embryos. Also note *Sox3* expression expanded in the brain of this embryo (K). Albino embryos injected unilaterally with *XSeb4R* mRNA, using *LacZ* mRNA as a tracer were cultured and fixed at neurula and at tailbud stages. In early fixed embryos, expression suppression was detected in 100% of embryos analyzed with A–P markers such as *Pitx2* (n = 32; M), *Otx2* (n = 35; N) and *Krox20* (n = 35; O). TUNEL assays showed a mild increase in apoptosis in about 40% of embryos (n = 35; P) and a massive apoptotic pattern was detected in 100% of tailbud embryos (n = 70) with a high magnification shown in panel R, the control uninjected overview sides are indicated in Q and *XSeb4R*-injected side in S. The injected sides (IS) are marked by X-gal staining in blue.

were monitored by real-time RT-PCR in ectodermal explants dissected from blastula stage embryos that were either overexpressing *XSeb4R* or were *XSeb4R*-depleted by injection of a previously characterized antisens morpholino oligonucleotides (XSeb4R-MO) (Boy et al., 2004; Souopgui et al., 2008). By this approach, evidence that *Sox3* is transcriptionally silent in naive ectodermal explants was established

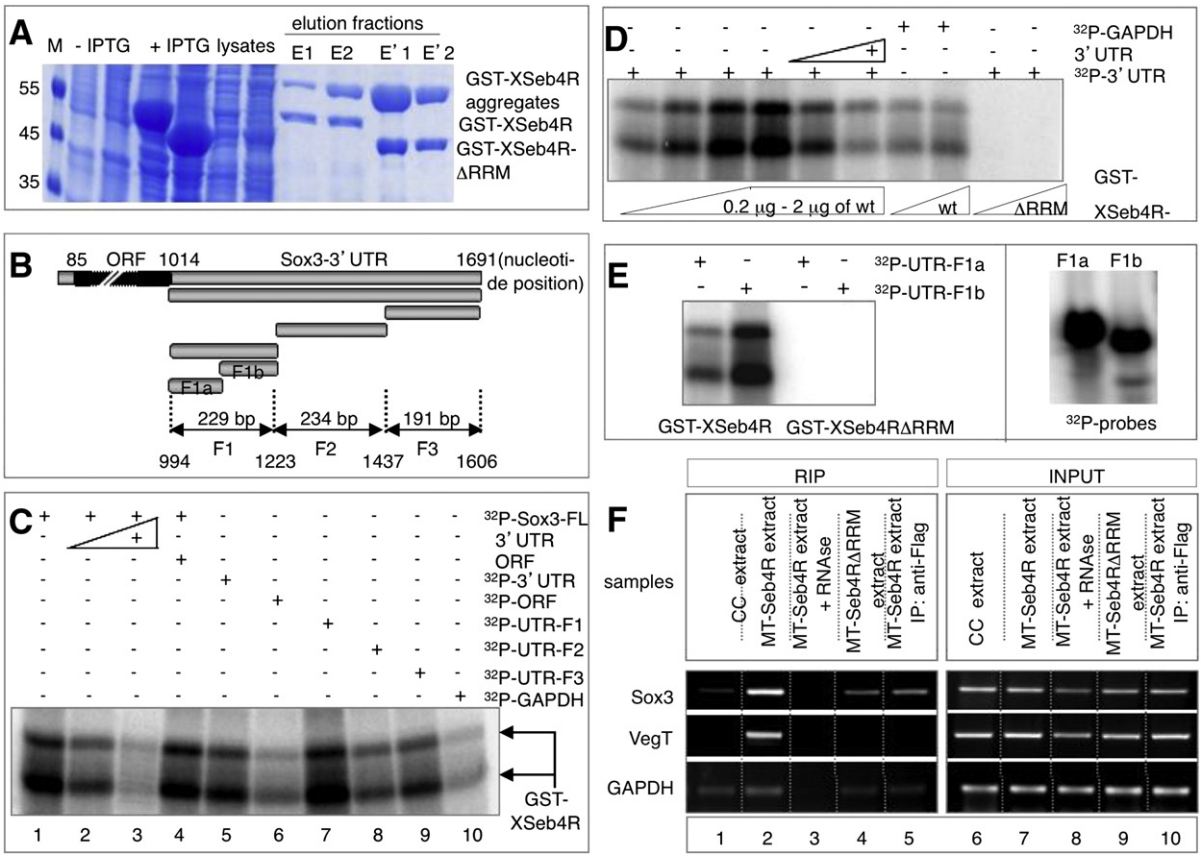

Fig. 3. *Sox3* mRNA is a direct binding target of the RNA-binding protein XSeb4R. (A) SDS-PAGE electrophoregram of coomassie stained gel. *XSeb4R* and *XSeb4RΔRRM* recombinant pGEX plamids were used to transform Bl21 bacterial cells. Cultures were induced (+IPTG) or not (−IPTG) for 4 h, harvested and sonicated in PBS supplemented with protease inhibitors. GST-XSeb4R and GST-XSeb4ΔRRM were purified on glutathione affinty columns. The corresponding eluted fractions are indicated. (B) Schematic representation of *Sox3* cDNA and the mutant constructs used in UV-crosslinking assays. Numbers indicate the nucleotide positions in the cDNA. (C) Electrophoregram of radioactive labeled GST-XSeb4R sample from UV-crosslinking. The two bands seen in purified proteins shown in A correspond to active GST-XSeb4R. As shown in lane 1, the full length (FL) *Sox3* mRNA probe binds to GST-XSeb4R. Signal of this interaction was competed with cold probe from the 3′UTR (lane 2, 1-to-1 and line 3, 1-to-10 molar ratio) but not from the ORF (lane 4, 1-to-10 molar ratio). Labeled probes from the 3′UTR (lane 5) but not from the ORF (lane 6) bind to GST-XSeb4R. Probes from 3 adjacent F1, F2 and F3 3′UTR show that F1 binds strongly compared to F2 and F3 (compare lane 7, 8, 9). A probe from *GAPDH* shows no significant interaction with GST-XSeb4R (lane 10). (D) The intensity of the 3′UTR but not GAPDH signal is proportional to the amount of protein used. The signal of the highest protein quantity was competed by unlabeled 3′UTR probe. GST-XSeb4RΔRRM shows no interaction with the 3′UTR probe. (E) Probe from the region spanning the F1b interacts with highest avidity to GST-XSeb4R. (F) Ribonucleic acid immunoprecipitation (RIP) coupled to RT-PCR shows that *Sox3* and *VegT* mRNAs are enriched in XSeb4R-RNP complexes (F, lane 2). Unspecific interactions, proportional to the amount of target RNA in the samples, were revealed in control assays (F, lane 1, 4 and 5). The RT-PCR products were analyzed on 2.3% agarose gels.

(Suppl. Figs. S1A, E). At the different time points analyzed, using *VegT* as a positive control (Fig. 4C), *XSeb4R* overexpression resulted in an increase of *Sox3* mRNA levels as compared with the uninjected control caps (Fig. 4B), suggesting increased RNA stability. However, semi-erratic *Sox3* mRNA levels were observed, probably as a result of zygotic Sox3 activation, since *XSeb4R* was reported to induce mesodermal and endodermal marker genes in animal caps (Souopgui et al., 2008), thereby leading to cap explant neurilization. Conversely, whereas MO-mediated XSeb4R protein knockdown led, as expected, to a strong reduction of *VegT* mRNA levels (Fig. 4C), the amounts of *Sox3* transcripts were, strikingly, elevated compared to the control at all analyzed time points (Fig. 4B). This increase in the levels of *Sox3* mRNA, results that were inconsistent with our model of *XSeb4R* function, was already observed at the time of cap explant dissection at blastula stage (t = 0 h, Fig. 4B). We then investigated if these elevated *Sox3* mRNA levels were really zygotic transcription independent. To this end, cap explants dissected from uninjected embryos or from those injected with XSeb4R-MO alone or in combination with α-amanitin and cultured for 5 h were analyzed by real-time RT-PCR. Results obtained show lower levels of *Sox3* mRNA in *XSeb4R*-depleted samples treated with α-amanitin campared to control and XSeb4R-MO cap explants (Fig. 4D), suggesting that *XSeb4R* is required in the turnover of the maternal *Sox3* transcripts but in a zygotic-transcription-dependent manner.

With the aim to provide an evidence that *XSeb4R* acts primarily on maternal *Sox3* mRNA, the origin of increased *Sox3* mRNA levels in *XSeb4R*-overexpressing samples was addressed. Capped-*XSeb4R* mRNA was injected alone or along with α-amanitin. As a control, this chemical was injected alone into the animal blastomeres of 4/8-cells stage embryos. Explants cultured for 5 h and analyzed by real-time RT-PCR revealed that α-amanitin treatment, unlike the zygotic gene such as *Epi. K.*, did not influence the levels of *Sox3* transcripts in control caps, thereby testifying not only their maternal origin (Suppl. Fig. S1E) but further suggesting that components involved in its turnover regulation are all maternally supplied. Moreover, α-amanitin treatment reduced the increased levels of *Sox3* mRNA from about five to three fold in *XSeb4R*-injected caps (Fig. 4E), suggesting a zygotic contribution of *Sox3* mRNA in *XSeb4R*-overexpressing samples and further supporting the semi-erratic pattern of the *Sox3* mRNA levels described above. Furthermore, the levels of *Epi. K.*, were reduced to less than half in *XSeb4R*-injected samples compared to naive caps and to almost zero in α-amanitin *XSeb4R*-caps (Fig. 4F). Thus, *XSeb4R* may also have a neural inducing potential in ectodermal explants, as was the case reported above in the brain (Fig. 2K).

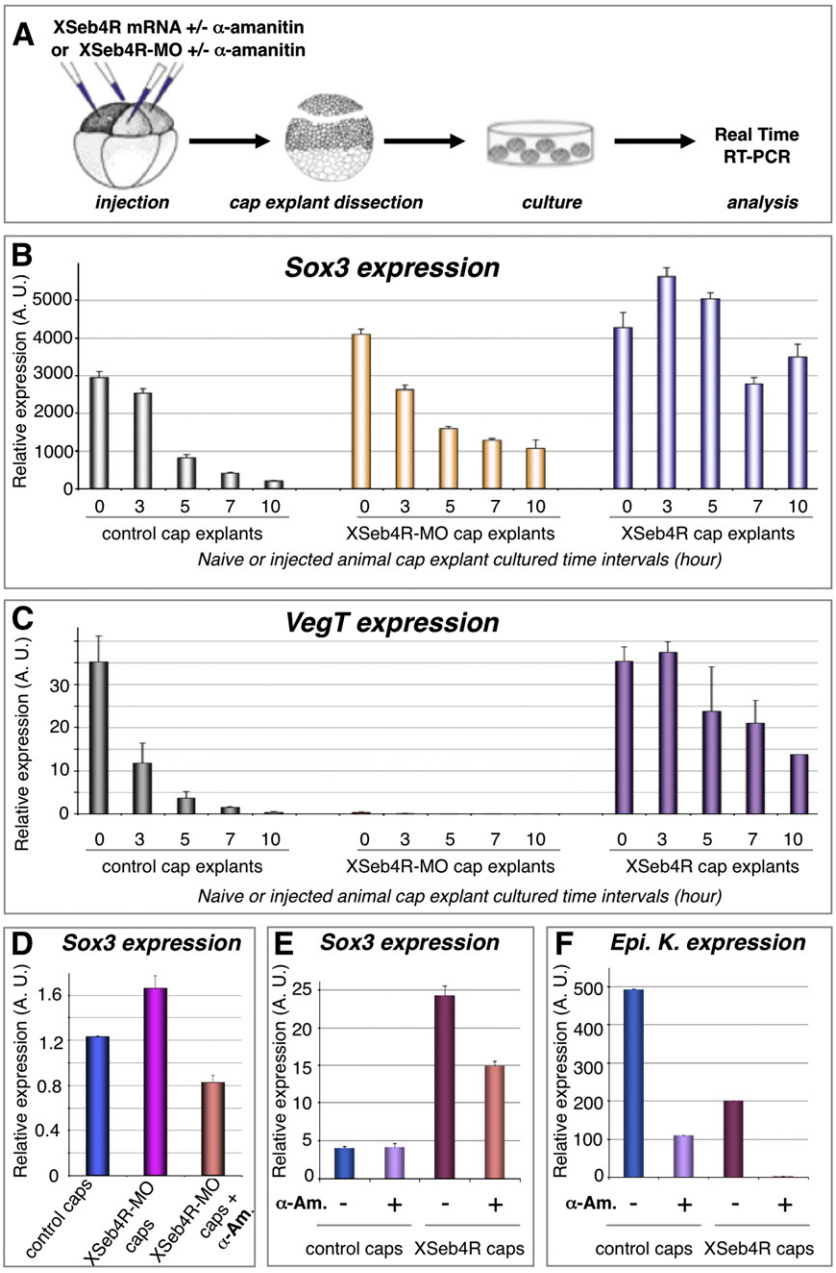

Fig. 4. XSeb4R stabilizes maternal *Sox3* mRNAs in ectodermal explants. Capped-*XSeb4R* mRNA and XSeb4R-MO were injected alone or in combination with α-amanitin into the four animal blastomeres of 4/8-cell stage embryos. The latter were cultured together with control uninjected embryos to blastula stage. Cap explants were dissected and cultured (A) at different time points as indicated and an average of 50 caps was collected per batch for mRNA analysis. The real-time RT-PCR results reveal significant increase of *Sox3* (B) and *VegT* (C) mRNA in *XSeb4R*-overexpressing caps. Note increased levels of *Sox3* but not *VegT* mRNA in XSeb4R-MO samples (B and C, yellow bars). This increase of *Sox3* mRNA levels was lost upon α-amanitin treatment (D). Alpha-amanitin treated cap explants were compared to naive or *XSeb4R*-overexpressing cap samples (E–F). Note the unchange levels of *Sox3* mRNA (E), unlike *Epi. K.* (F), in treated and untreated cap explants. In *XSeb4R*-injected caps, the levels of Sox3 mRNA, but not *Epi. K.*, reamined significantly high compared to the control untreated naive caps (E and F). The RNA quantification was done in duplicate or triplicate and the standard deviation is added to the histogrammes. Data are expressed relative to *GAPDH* mRNA. Note a use of different scales in graphics from panels B to F.

XSeb4R regulates maternal Sox3 mRNA translation

As a posttranscriptional regulator of gene expression, *XSeb4R* may also modulate *Sox3* regulation at the translational level. This was indeed the case, as *MT-Seb4R* overexpression in animal caps robustly activated *Sox3* translation (Fig. 5A). At the time of explant isolation (blastula stage) which corresponds to the cap culture time t = 0 h in Figs. 4B and C, XSeb4R-MO injected samples, although containing significantly more *Sox3* mRNA than the control caps (Fig. 4B), revealed instead a lower amount of Sox3 protein compared to the control uninjected caps (Fig. 5A; compare lane 1 and 3), suggesting that *XSeb4R* is required for maternal *Sox3* mRNA translation. To strenghten this possibility, immunostaining on cap explants from unilaterally *XSeb4R* and XSeb4R-MO injected embryos were performed. The analysis revealed strong Sox3 protein expression in *XSeb4R*-overexpressing cells (Fig. 5C). Here, a high magnification image indicates a smear like pattern of Sox3 expression on the injected side of the cap explants, indicating that cells are filled up with Sox3 proteins. Conversely, XSeb4R-depleted cap explants, marked by the cytoplasmic LacZ/red X-gal staining (Turner and Weintraub, 1994), did not reveal a significant change in Sox3 production (Fig. 5D), although the amount of *Sox3* mRNA was expected to be higher.

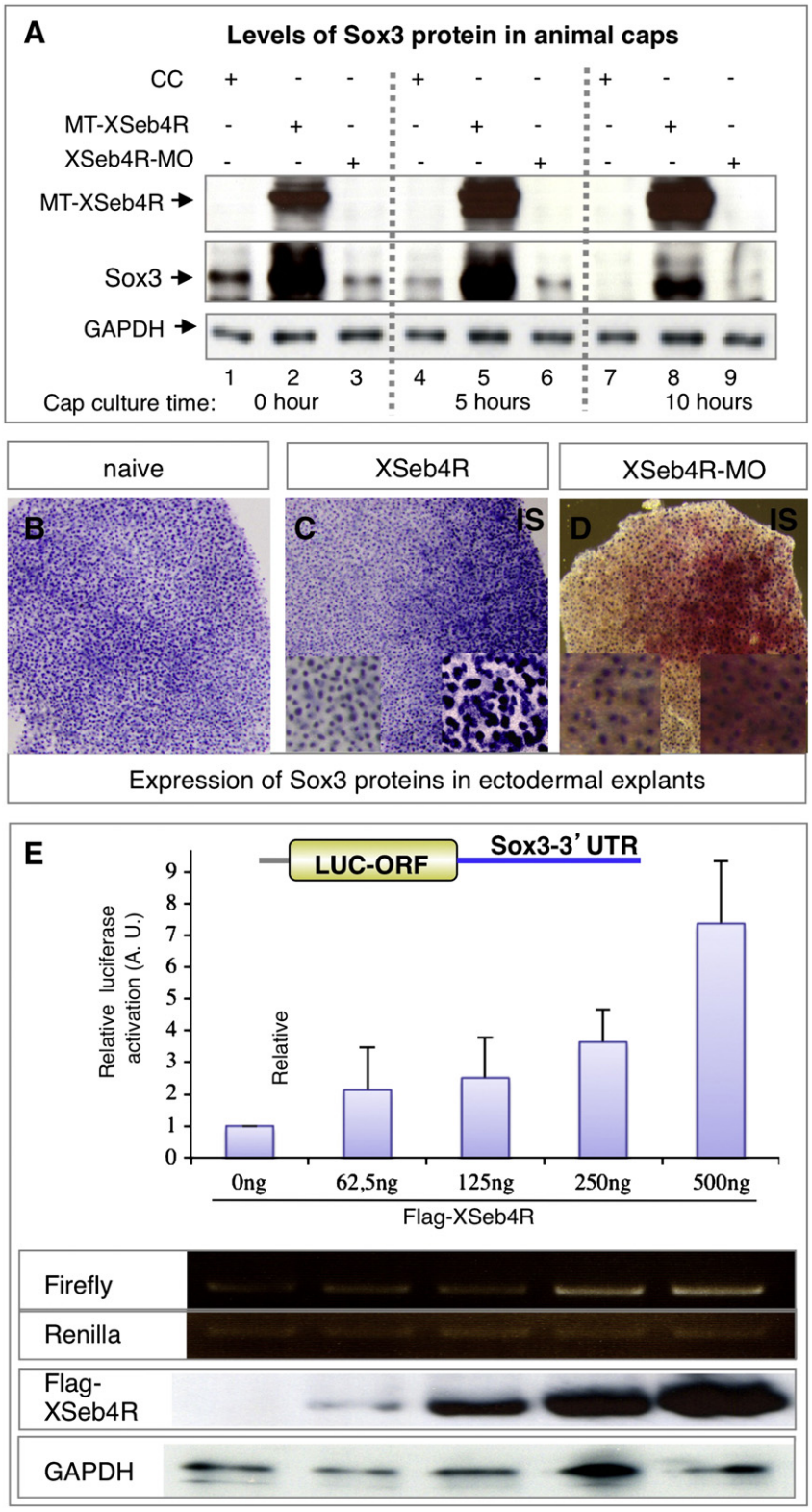

Fig. 5. *XSeb4R* strongly activates maternal *Sox3* translation. (A) Western blot analysis of *Sox3* translation in caps from *MT-XSeb4R* or XSeb4R-MO injected embryos, using an anti-Sox3 antibody. GAPDH antibody was used as loading control. Note a progressively increased amount of Sox3 signal in MT-XSeb4R overexpressing caps at the equivalent of blastula, t0 (lane 2) and gastrula, t5 (lane 5), but not at neurula, t10 (lane 8) stages of explant development. Observe a decrease in the level of Sox3 signal at the time of explant dissection (t0) in *XSeb4R* depleted caps (lane 3) compared to the control (lane 1). (B–D) Ectodermal explants dissected at the blastula or gastrula stage from embryos that were injected unilaterally with *XSeb4R* mRNA or XSeb4R-MO were stained with an anti-Sox3 antibody. Caps from control uninjected embryos show a uniform staining pattern (B; 100%, n = 50); expanded signals are observed on the injected side of explants from *XSeb4R*-overexpressing embryos (C; 100%, n = 50). Sox3 protein translation was not significantly affected in *XSeb4R*-depleted caps (D; 100%, n = 50; high magnification: compared signals in the cells stained in red and unstained areas). (E) Sox3-3′UTR Luciferase reporter was analyzed in HEK293 cells. All firefly luciferase values were normalized to renilla luciferase. Shown are the relative luciferase expression activation; note that *XSeb4R* stimulates *LUC-Sox3-3′UTR* in a concentration dependent manner. Four independent transfections revealed similar trends. Also observe increased firefly but not renilla luciferase mRNA stability in response to *XSeb4R*, as tested by RT-PCR. The increased amount of Flag-XSeb4R protein in transfected cells is added, with GAPDH as a protein loading control.

The results described above led us to predict that fusion of the *Sox3*-3′UTR to the luciferase open reading frame (ORF) should result in a reporter construct that is translationally activated by XSeb4R protein. To experimentally address this question, the luciferase reporter response was quantified in transfected cells. We found that a *LUC-Sox3*-3′UTR reporter, indeed, responded in a dose-dependent manner to *XSeb4R* stimulation (Suppl. Fig. S3). Importantly, *XSeb4R* activates *LUC-Sox3*-3′UTR in HEK293 cells in a dose dependent manner (Fig. 5E). However, the increased luciferase activity in response to *XSeb4R* co-transfection was associated with increased stability of the corresponding mRNA reporter. Interestingly, the mRNA from the renilla luciferase construct, used as an internal control, did not reveal a detectable expression variation in response to *XSeb4R* co-transfection (Fig. 5E), suggesting that *XSeb4R* acts on specific target RNAs.

XSeb4R is required in primary ectoderm specification in Xenopus embryos

The maternal Sox3 proteins act as an antagonist of the TGFβ responses during germ layer formation in *Xenopus*. It was shown that the inhibition of Sox3 binding to target DNA sequence on the mesodermalizing genes (Zhang et al., 2003) by injecting a specific anti-Sox3 antibody led to a disruption of the normal animal–vegetal patterning of mesodermal and endodermal marker gene expression. The ectoderm–mesoderm boundary was shifted towards the animal pole while the expression levels of the endodermal gene Sox17 was increased (Zhang et al., 2004). Hence, if the ability of *XSeb4R* to regulate maternal *Sox3* mRNA serves an essential role in germ layer specification, *XSeb4R* depletion in the animal blastomeres should result in a phenotype reminiscent to that of the anti-Sox3 antibody. To address this possibility, XSeb4R-MO was unilaterally injected into the animal hemisphere of 4-cell stage embryos. These embryos, cultured and fixed at stage 10.5 or st.14, were analyzed by WMISH targeting mesodermal marker genes. As expected, XSeb4R-morphant embryos revealed an expansion of the zygotic *VegT* (*Apod*) expression into the presumptive ectoderm (Fig. 6B). The injeceted embryos, fixed at a later stage of development, revealed an expanded *Xbra* expression (Fig. 6C), suggesting that this mesodermal fate induction was not transient.

To further support the possibility that *XSeb4R* plays an active role in ectoderm specification, the animal cap assay was employed. Ectodermal explants develop into atypical epidermis marked by the expression of specific genes such as epidermal keratins. In these assays, the ectodermal explants dissected from XSeb4R-MO injected embryos and cultivated till the late gastrula stage of development were analyzed by real-time RT-PCR. Results obtained revealed a strong ectopic expression of *Xbra* at the expenses of *Epi. K.*, an epidermal specific molecular marker in *XSeB4R*-depleted cap explants (Fig. 6D), suggesting that *XSeb4R*-depleted ectodermal progenitors are permissive to mesodermalizing factors.

All together, these data strongly suggest that the maternal *XSeb4R* expression in the animal pole derived progenitor cells correlates with its function, via at least in part the TGF-β antagonist *Sox3*, in primary ectoderm specification in *Xenopus*.

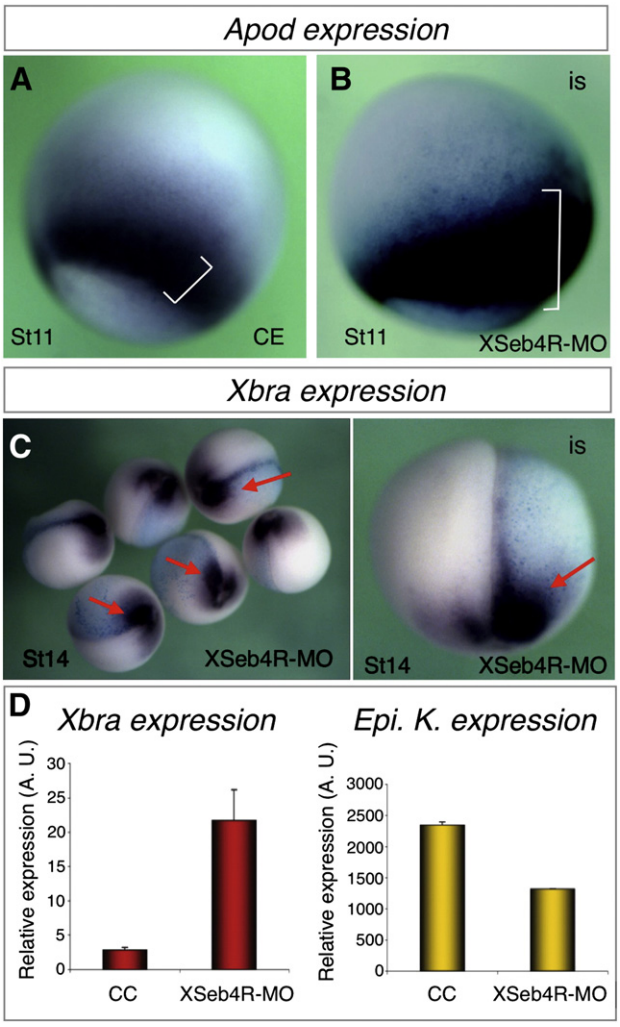

Fig. 6. *XSeb4R* regulates the levels and expression boundaries of mRNAs encoding germ layer specification markers. XSeb4R-MO was injected into the animal hemisphere of one blastomere of two- or four-cell stage albino embryos and the latter were analyzed by WMISH at gastrula and neurula stages, using the indicated probes. The control embryo (CE) shows a ring-like expression pattern around the blastopore and the width is indicated (A). The injected embryo, oriented vegetal pole down, revealed an upward expression expansion (46%, n = 60) in the presumptive ectoderm and the width is indicated (B). Xbra analysis in neurula stage morphant embryos revealed an expanded expression in 80% of the total embryos (n = 45). Caps from CE or from embryos injected with XSeb4R-MO in the four micromeres at 4/8-cell stage were analyzed by real-time RT-PCR targeting mesodermal marker *Xbra* and the ectodermal gene *Epi. K*. XSeb4R protein knockdown induces *Xbra* expression and a repression of *Epi. K*. expression (C). Data are expressed relative to *GAPDH* mRNA.

Discussion

In this study we report on the function of the RNA-binding *XSeb4R* in *Xenopus* ectoderm formation. We provide evidence that the maternal *XSeb4R* mRNA degradation prior to that of maternal *Sox3* mRNA is associated with the posttranscriptional regulation of *Sox3* by *XSeb4R* during primary ectoderm specification. XSeb4R protein binds directly and specifically to the 3′UTR of *Sox3* mRNA. Forced expression of *XSeb4R* in ectodermal progenitors abnormally stabilizes maternal transcripts, including *Sox3*, resulting in impaired ability of these progenitors to respond to factors involved in brain patterning, followed by their eventual loss by apoptosis. In the animal caps system, *XSeb4R* gain- and loss-of-function modulate the translation of maternal Sox3 mRNA. *XSeb4R* depletion in ectodermal progenitors leads to an expansion of the mesodermal marker genes into the presumptive ectoderm.

XSeb4R protein interacts directly with various mRNA targets

The RNA-binding protein *XSeb4R* was previously reported to bind specifically to the 3′UTR of the T-box transcription factor *VegT* (Souopgui et al., 2008). However, blastula stage embryos contain equal amounts of *XSeb4R* transcripts in the animal pole and in the vegetal mass. If *VegT* were the unique mRNA target of *XSeb4R* their expression profile would have been identical. The strong expression of *XSeb4R* in the animal pole derived progenitors is indicative of the existence of other mRNA targets and, therefore, of a different

XSeb4R function in these cells. By use of UV-crosslinking method we showed that *Sox3* was another direct target of *XSeb4R*. Consistently, *RNPC1*, the human homolog of *XSeb4R* binds directly to *p21*, *p63* and *p53* mRNAs (2011; Shu et al., 2006; Zhang et al., 2010). Other RNA-binding proteins were reported to interact with multiple target transcripts. For example, thousands of direct and functional *HuR* targets have recently been reported (Lebedeva et al., 2011). Quite often, consensus sequences explain the direct interaction of an RNA-binding protein with many targets. For example, transcripts containing AU-rich elements (ARE) in their 3′UTR are able to interact directly with RNA-binding proteins harboring RRM domain in their sequence structure (Li et al., 2010). We, however, did not identify a conserved motif in *Sox3* and *VegT* mRNAs, although they contained several stretches of GU-rich sequences in their 3′UTR. As some RNA-binding proteins interact preferentially with secondary structure (Li et al., 2010), this could be the case for *XSeb4R*. It would be necessary to address experimentally this, in order to understand how this RNA-binding protein achieves affinity with the various target mRNAs. As the first hint, the formation of specific RNP-complexes with different protein partners would explain *XSeb4R* ability to select and differentially regulate its mRNA targets.

XSeb4R regulates the maternal but not the zygotic Sox3 expression

The B1-type HMG Sox protein, *Sox3* is mostly known as a pan-neural marker in *Xenopus* (Klisch et al., 2006; Penzel et al., 1997). The ectopic expression of *Sox3* in neurula stage embryos following overexpression of *XSeb4R* could stem a neural inductive function of *XSeb4R*. However, this phenotype was proven to be the results of increased stability of the maternally expressed *Sox3* transcripts following *XSeb4R*-forced expression. Consistently, the expression profiles of *XSeb4R* and *Sox3* overlap at blastula stage of development but diverge during neurogenesis. The binding of XSeb4R protein to *Sox3* mRNA likely correlates only with the early (maternal mRNA) but not the late (zygotic mRNA) function of *Sox3*. Our data showing huge amount of Sox3 protein in ectodermal explants from *XSeb4R*-injected embryos could, therefore, be reminiscent of the endogenous activities of this RNA-binding protein.

XSeb4R acts at the posttranscriptional levels on Sox3 mRNA stability and translation

RNA-binding proteins are key regulators of gene expression directly responsible for the total amount of cellular protein during development and in disease expression. These molecules influence either indirectly the amount of a given protein, acting on the stability of the transcripts or directly, interacting with the mRNA and stimulating translation. We previously showed that *XSeb4R* could play such direct and indirect roles on *VegT* during germ layer formation in *Xenopus* (Souopgui et al., 2008). Recently, *RNPC1* was reported to bind to the 5′UTR of *p53* mRNA, thereby repressing its translation in cancer cells (Zhang et al., 2011). The fact that our experimental data does not only show maternal *Sox3* mRNA stabilization by *XSeb4R* associated with huge amount of Sox3 protein in animal cap assays but also show that the luciferase activity is increased following reporter RNA stabilization by *XSeb4R* in HEK293 cells strongly suggest that *XSeb4R* plays a role in the stoichiometry of Sox3 protein during ectoderm specification. Whether, XSeb4R acts directly on Sox3 translation remains unclear from our data. However, the observation that XSeb4R knock down led to an increased amount of *Sox3* transcripts without production of more Sox3 proteins supports the hypothesis that XSeb4R is a component of the translational machinery that regulates *Sox3* expression during germ layer formation in *Xenopus*. Remarkably, XSeb4R depletion by use of morpholino did not erase completely Sox3 protein signals as revealed by Western blot and histoimmunochemistry analysis, suggesting that Sox3 translation was effective before morpholino injection. If so, it could be further speculated that Sox3 proteins are very stable during early development.

XSeb4R model of function during early embryonic development

In *Xenopus*, the early embryonic development, as well as the initial cell allocation to the different germ layers is mediated by the determinants encoded by the maternal transcripts (Heasman, 2006). The zygotic expression of some of these determinants is restricted to specific tissues and cells. For example, the maternal *VegT* transcripts are mostly inherited by the vegetal-derived endodermal progenitors, while *Apod*, the zygotic form of *VegT* is expressed in the mesodermal cells and later in the sensory neurons (Zhang and King, 1996; Stennard et al., 1996). In order to avoid interference in their activities the mRNAs encoding the maternal proteins are systematically degraded as from the onset of the corresponding zygotic transcription. The spatio-temporal regulation of the maternal transcripts degradation is modulated by microRNAs and RNA-binding proteins (Schier, 2007). Consistently, we reported recently that *XSeb4R* regulates the stability of *VegT* mRNA (Souopgui et al., 2008). However, while the stability of *VegT* transcripts was found to be *XSeb4R* dependent, our findings and model of *XSeb4R* function in this work are deeply challenged. In both gain- and loss-of-function experiments the levels of *Sox3* mRNA were increased compared to the samples form uninjected embryos. The augmented amount of *Sox3* mRNA in *XSeb4R*-depleted explants was detected at the time of animal cap dissection at blastula stage and was lost upon inhibition of the zygotic transcription. This strongly indicates that *XSeb4R* is required for the turnover of maternal *Sox3* transcripts. Consistent with the role of microRNAs in the degradation of maternal transcripts, we speculate that *XSeb4R* may participate in the biogenesis of a microRNA of the zygotic origin that regulates Sox3 mRNA turnover. Since a preMBT transcription activation was recently reported in *Xenopus* (Skirkanich et al., 2011), the zygotic expression of this suspected microRNA should also be activated before blastula stage of development. Overall, we suggest a model of *XSeb4R* function whereby binding to the 3′UTR of its target mRNAs, XSeb4R protein prevents their degradation (case of *VegT* and *Sox3*; also see Koebernick et al., 2010) and a second scenario where XSeb4R protein modulates the processing/maturation of specific microRNAs to regulate the turnover of its RNA targets (case of *Sox3*; also see Trabucchi et al., 2009). It is of prime interest, therefore, to investigate whether *XSeb4R* regulates directly or via protein partners the biogenesis of microRNAs during early embryonic development in *Xenopus*.

XSeb4R is an active component of the gene network required for ectoderm formation in Xenopus

The optimal ectoderm formation is a result of antagonistic activities between signaling cues emanating from the equator of blastula stage embryos and the ectodermal determinants in *Xenopus* (Dupont et al., 2005; Mir et al., 2007; Sasai et al., 2008a; Suri et al., 2005). However, these antagonistic activities have been mainly interpreted as prerequisites in the germ layer boundary delimitation. The importance of such antagonistic activities within the developing ectoderm has not been addressed. Most of the mesodermal inducing factors, including *Xenopus nodal related* (*Xnr*) genes are *VegT* targets. Transcripts encoding members of the TGFβ/nodal gene family have been identified within the ectodermal explants but their roles in this tissue remain elusive. Interestingly, *Sox3* was reported to act as a transcriptional repressor of *Xnr5* and *Xnr6* in both whole embryo and animal caps injected with *VegT* mRNA (Zhang and Klymkowsky, 2007; Zhang et al., 2003). Moreover, maternal Sox3 protein induces the expression of ectodermal determinants, *Ectodermin*, *FoxIe*/*Xema* and *Coco* (Zhang and Klymkowsky, 2007). The maternal *Sox3* mRNA

is regulated at the posttranscriptional levels by *XSeb4R*. In doing so, *XSeb4R* functions as a pro-ectodermal factor. As evidence, *XSeb4R* depletion expands the expression of mesodermal marker genes such as *Xbra* and *Apod* in both the embryos and ectodermal explants. However, it remains elusive how and why *XSeb4R* regulate simultaneously members of the gene families belonging to two antagonistic signaling pathways, such as *VegT* and the maternal *Sox3*.

Material and methods

Embryo manipulation, explant dissection, in situ hybridization, TUNEL assay and immunohistostaining

Wild-type and albino *Xenopus* embryos were obtained by hormone-induced egg laying and *in vitro* fertilization using standard procedures. Embryos were staged as described by Nieuwkoop and Faber (Gherzi et al., 2010). Whole mount *in situ* hybridization was done as described (Kumasaka et al., 2003). Cap explants were dissected at the blastula stage or early gastrula using either an adapted eyelash unit or forceps. Sox3 immunostaining was done as previously reported (Zhang et al., 2003). Cell proliferation targeting phosphohistone H3 (PPH3) was performed as described (Saka and Smith, 2001). TUNEL assays were done as reported (Hensey and Gautier, 1998). Transcription inhibition was performed as described (Lee et al., 2001). All injections were performed at least twice and the effects indicated correspond to one representative experiment.

DNA constructs, RNA synthesis, oligonucleotides and protein production

Expression plasmids were generated by PCR and cloning of the PCR products into the EcoRI and XhoI sites of a pCS2+ vector and its derivatives were done as indicated below. The *Sox3* 3′-UTR was cloned (Bam*HI*/Not*I*) in the pBKCMVLuc vector (Stratagene) downstream of firefly luciferase, generating *Luc-Sox3* 3′-UTR. Renilla luciferase contructs was described earlier (Souopgui et al., 2008). Templates for the different 3′UTR fragments were generated by PCR with SP6-promoter sequence fused to the forward primer (Table 2). For *in vitro* transcription, DNA constructs were linearized with NotI. Capped-mRNAs were produced using the mMessage mMachine kit (Ambion). For *in situ* hybridization or UV-crosslinking, Dig- or ^{32}P-UTP labeled probes were produced by making use of the appropriate RNA polymerases. The purification of GST-tagged XSeb4R was achieved as described (GST Gene Fusion System, Amersham Biosciences). XSeb4R-MO and VegT-MO oligonucleotides were described previously (Boy et al., 2004; Heasman et al., 2001).

Table 1
RT-PCR and Q-PCR primers.

Gene	Primers
Epi.K	F: GTGATAGCAATGGCCTTCGT
	R: CTCACTTTGCCAGCACTCG
Firefly	F: AGGGCGGAAAGTCCAAATTG
	R: TGAAAGCCCAACACATCATC
GAPDH	F: TAGTTGGCGTGAACCATGAG
	R: GCCAAAGTTGTCGTTGATGA
Renilla	F: ATCGGACCCAGGATTCTTTT
	R: ACTCGCTCAACGAACGATTT
Sox3	F: AGACACTTACGCGCACATGA
	R: TACCTGTGCTGGATCTGCTG
VegT	F: AGAAACTGCTGTCGGGAA
	R: CGGATCTTACACTGAGGA
Xbra	F: GAATGGTGGAGGCCAGATTA
	R: TTCATTCTGGTATGCGGTCA
XSeb4R	F: TTCTGAAACGGGATCAGTCC
	R: CTGCAATGCAACGTCATACC

Table 2
Primer pairs for UV-CL gene fragments.

Gene fragments	Primers
Sox3-FL	F: CATACGATTTAGGTGACACTATAGAACACTAGTGGAAAGGAAAGT
	R: GAGTATGTCGTTTACTTTCATGTC
Sox3-ORF	F: CATACGATTTAGGTGACACTATAGAATGTATAGCATGTTGGACAC
	R: TATATGTGAGTGAGCGGTAC
Sox3-3UTR	F: CATACGATTTAGGTGACACTATAGAGTACCGCTCACTCACATATAACAC
	R: GAGTATGTCGTTTACTTTCATGTC
Sox3-3UTR-F1	F: CATACGATTTAGGTGACACTATAGAGTACCGCTCACTCACATATAACAC
	R: TGAAAGCCCAACACATCATC
Sox3-3UTR-F2	F: CATACGATTTAGGTGACACTATAGAGATGATGTGTTGGGCTTTCA
	R: GTCCTGTTTGCGACCCGCAC
Sox3-3UTR-F3	F: CATACGATTTAGGTGACACTATAGAGCTGTGCGGGTCGCAAACAGGAC
	R: GAGTATGTCGTTTACTTTCATGTC
Sox3-3UTR-F1a	F: CATACGATTTAGGTGACACTATAGAGTACCGCTCACTCACATATAACAC
	R: CACCCTGATTCATTGTCCTC
Sox3-3UTR-F1b	F: CATACGATTTAGGTGACACTATAGAGAGGACAATGAATCAGGGTG
	R: TGAAAGCCCAACACATCATC
GAPDH-3UTR	F: CATACGATTTAGGTGACACTATAGAGTTGATAAACCAAATCAAGTGACTAC
	R: AGGAACACAGAAACAGTGAC

RT-PCR and Real-time RT-PCR (Q-PCR) analysis

Total RNAs were extracted using the RNAspin Mini RNA isolation kit (GE Healthcare). All samples were tested for DNA contamination by 30 cycle PCR amplification using histone H4 primers (Niehrs et al., 1994). Complementary DNAs were synthesized with iScript cDNA synthesis kits (Biorad). RT-PCR was done according to Gene Amp RNA PCR kit (Perkin Elmer). Real-time RT-PCR was carried out using the Step One Plus Real Time PCR system (Applied biosystems) with Q-PCR core kits for SYBR Green I (Eurogentec). The primer pairs used are resumed in Table 1. Samples were normalized with *Xenopus GAPDH*. All experiments were repeated at least two times with each assay performed in duplicate or triplicate.

UV-crosslinking assays

In vitro UV-crosslinking reactions were performed as previously described (Souopgui et al., 2008). ^{32}P-labeled probes were incubated with optimized amounts of GST-XSeb4R or GST-XSeb4RΔRRM proteins in a buffer containing or not the competitor. Probes were normalized based on their cpm.

RNA- and protein-immunoprecipitation, and Western blot

For the RNA-immunoprecipitation (RIP), MT-XSeb4R or MT-XSeb4RΔRRM mRNA (200 pg/cell) was injected into the animal blastomeres of 4/8-cell stage embryos that were cultured to blastula stage of development. Explants were dissected and processed. We first ensured by Western blot that the tagged-protein of interest was produced (data not shown). Then, MT-XSeb4R extract was subdivided into three aliquots. After treatment of an aliquot with RNase, an anti-MT antibody was used to immunoprecipitate XSeb4R-RNA complexes from the latter together with the second aliquot, whereas the third aliquot was processed with an anti-Flag antibody as a control. Next, the immunoprecipitated complexes were treated with RNase-free DNase to digest possible trapped genomic DNAs. The associated target RNAs were purified and used for cDNA synthesis. For the input controls, 5% of each individual sample aliquot were saved for direct RT-PCR analysis.

Cell transfection and luciferase reporter assays

HEK293 cells were transfected with *LUC-Sox3-3′UTR* reporter alone or together with optimized concentrations of Flag-tagged *XSeb4R*

(in pCS2 +). *Renilla Luciferase* reporter (0.5 ng) was co-transfected to normalize the data. Transfected cells, cultured for 24 h, were aliquoted into three portions for luciferase, Western blot and RT-PCR analyses, respectively. For the luciferase quantification, cells were homogenized in 0.5 volume of 1× passive lysis buffer (Promega). Ten microliters diluted 10× of each lysate was assayed using the Dual Luciferase Reporter system (Promega) in a LB960 CENTRO Microplate Luminometer (BERTHOLD Technologies). Each transfection was repeated at least three times.

Supplementary materials related to this article can be found online at doi:10.1016/j.ydbio.2011.12.040.

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